SYBR GREEN I
ASSAY PROTOCOL: ABI PRISM®

This protocol is adapted from an Applied Biosystems protocol by James Cherry to meet the needs of 7900HT SDS.
This protocol is for use with Applied Biosystems control Gene Systems and Housekeeping Gene Systems. For additional technical inquiries, contact Technical Service at 800-762-4001 or tech.support@appliedbiosystems.com

BEFORE STARTING THE EXPERIMENT

Thermophilic Taq polymerase

Applied Biosystems' Master mix and Ampli Taq Gold. Other Thermophilic Taq Polymerases maybe used; contact tech support.

PCR Reaction Vessel

Perform the assay with optically clear amplification tubes and caps or an optically clear PCR plate.

Experimental Design

The required controls for each target of interest are the No Target Control and the Target Template Dilutions.

No Target Control (NTC)

This control is used to determine the limits of PCR sensitivity and tests reactions for possible background that may occur during amplification. The statistical significance of lowest detectable signal is determined by employing the threshold cycle (C_t) of NTC. This control also assesses the generation of primer-dimer PCR artifact or amplicon contamination of a kit reagent.

Target Template Dilutions

Generation of a standard curve is required with each experiment and for each target of interest (i.e. one for each housekeeping target and one for each target).

Serial 10-fold dilutions of the Target Template (10^6 to 10^1 copies per reaction) are amplified. Under the experimental conditions described in this bulletin, it is recommended that target dilutions be run in duplicate for increased accuracy.

Experimental Samples
Dilutions of experimental samples are generally not required. Run at least duplicates of all experimental samples.

REAL TIME ASSAY PROTOCOL

Step A. Thermocycling Conditions

1. Enter the parameters listed in Table 1 into the thermocyler.

Table 1: PCR Program

Stage	**1**	**2**		**3**		
		Ampli Taq Gold		***Repeat: 40-45 cycles***		
		Activation	**Melt**	**Anneal/Extension**		
Step			**(1)**	**(2)**	**(3)**	**(optional)**
Temperature	**50°C**	**95°C**	**95°C**	**60°C**	**72°C**	
Time (min.)	**2:00**	**10:00**	**00:15**	**1:00**	**1:00**	

Note: a Dissociation Curve must be done with the use of SYBR Green
95°C/15seconds (100%), 60°/15 seconds (100%), 95°C/15seconds (2%)
Data collection for the dissociation curve is collected during the ramp time to: 95°C/15seconds (2%)

2. Typically, ROX fluorescence is detected along with the signal of the fluorophores used to label the amplified product.

Step B. Assay Procedure

1. Add 2 µl of H_20 to tubes designated as No Target Controls.

2. Using pipettes designated only for aliquotting DNA, prepare an initial working stock solution. Prepare 1:10 serial dilutions of the initial stock solution.

Table 2: Preparing Target Template Dilutions

	Stock Solution	H_20
1	Working stock	
2	5 µl of #1	45 µl
3	5 µl of #2	45 µl
4	5 µl of #3	45 µl
5	5 µl of #4	45 µl
6	5 µl of #5	45 µl
7	5 µl of #6	45 µl

3. Aliquot 2 µl of the serially diluted control template (in duplicate) and experimental samples (duplicate or triplicate).

4. Prepare the PCR "Master Mix" using the reagents (equilibrated at room temperature) listed in Table 3.

Table 3: Preparation of PCR "Master Mix"

Components of "Master Mix"	Volume per Reaction
DH_2O	**9.5 µl**
10uM Mu GAPDH Forward Primer	**0.25 µl**
10uM Mu GAPDH Reverse Primer	**0.25 µl**
2X Taq Man PCR Master Mix	**12.5 µl**
Total Volume	**22.5 µl**

Note: Volume of Primers vary. This master mix is for only Mu GAPDH Control Kit.

5. Place tubes in thermocycler and begin amplification.

EXAMPLE OF DATA ACQUISITION AND ANALYSIS

1. An assay using Taq Man Vic Probe Control Kit was performed with serial dilutions of Murine GAPDH Target cDNA. All experimental procedures are as previously described. Real-time fluorescence was measured using the ABI PRISM® 7900.

2. A standard curve was generated by plotting the $\log_{10}$ [target dilution] of Mu GAPDH control template on the X axis against the C_t value from serial dilutions of Mu GAPDH target DNA on the Y axis (Figure 1). Standard curve, linear equation, and correlation coefficient (R^2) are automatically computed with the PRISM® software.

a. The standard curve is linear over 6 logs (10^1 to 10^6 dilutions) with correlation coefficient (R^2) of 0.992 Standard curve equation is Y= -3.297 X + 39.493

b. Relative copy # of unknown samples are determined according to standard curve equation (data not shown).

c. An amplification plot of the Control Template Dilutions (10^6 to 10^1 target cDNA per reaction) and No Target Control is presented in Figure 2. No signal is observed for the No Target Control; therefore, all C_t values for target dilutions are significant.

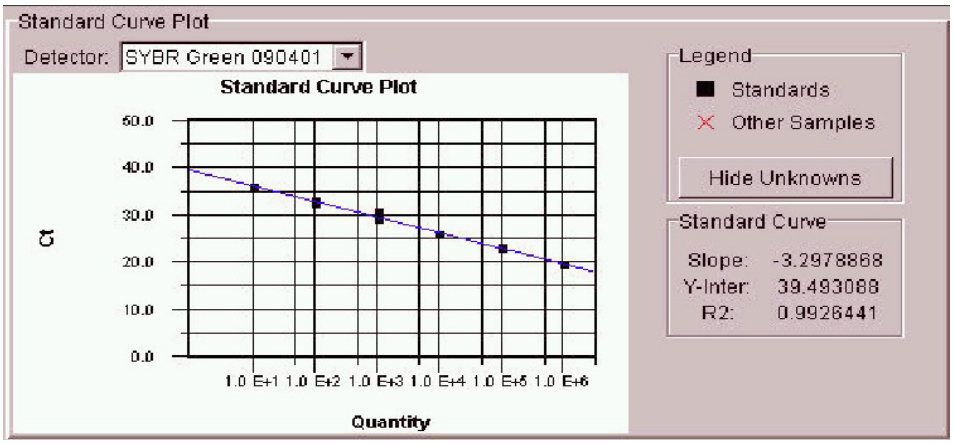

Figure 1 Standard Curve

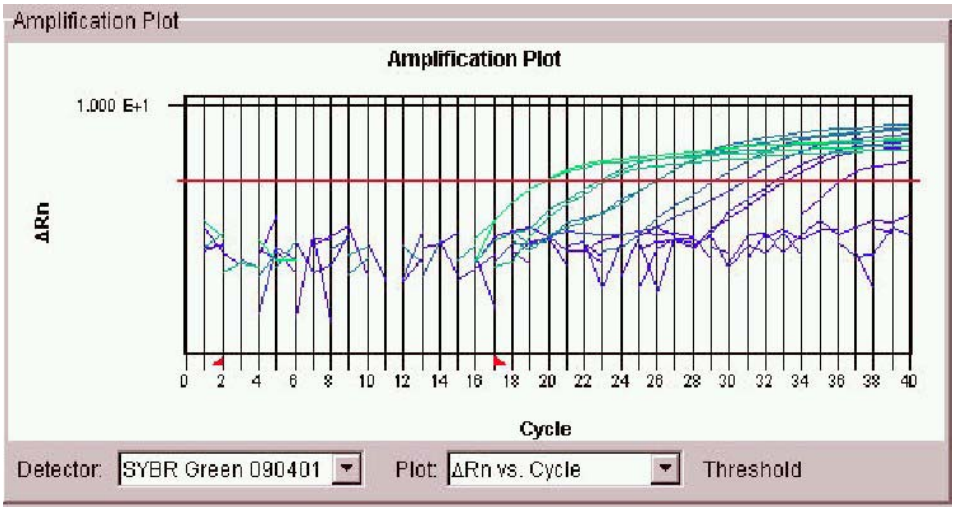

Figure 2 Amplification Plot

TROUBLESHOOTING

A. No amplification is observed.

Potential problem: PCR amplification is not initiated.

Recommendations:

1. Recheck the addition of kit reagents. Were the correct amounts of each component added and mixed? Was 2X Master Mix included?
2. Recheck the thermocycler for proper temperature and time settings. Is the thermocycler cycling at 95°C/15 seconds and 60°C/1 minute for 40 cycles? Is the initial 50°C/2 minutes and 95°C/10 minutes denaturation step included?
3. Check the Taq polymerase to see if it is active.

B. No template Control shows amplification (positive C_t is obtained for NTC).

B1. Potential problems: Primer-dimer PCR artifacts.

As is anticipated with a PCR-based assay, some unavoidable PCR artifacts are expected even when the optimal assay conditions are employed.

Recommendations:

1. While primer-dimer reduces target sensitivity, C_t values for Target Template Dilutions and Experimental Samples that are 4 cycles lower than the No Target Control are statistically significant. A four-cycle difference approximately equals a 10-fold difference in initial target concentration.
2. Use a "Hot Start" Taq polymerase in order to improve specificity. 2X Master Mix *Taq* polymerase (Applied Biosystems) is recommended. "Hot start" enzymes available from other sources may require assay optimizations.
3. Do not amplify over 40-45 cycles.
4. Optimize the amplification conditions by increasing annealing/extension temperature to 63°C.

B2. Potential problems: PCR carry-over contamination.

Recommendations

1. Use fresh aliquots of assay reagents
2. Follow these recommendations (Laboratory Set-up and Precautions). PCR racks are the most likely source of PCR carry-over contamination. Decontaminate the racks UV 30 minutes prior to use.